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Cathepsin E–Deficient Mice Show Increased Susceptibility to Bacterial Infection Associated with the Decreased Expression of Multiple Cell Surface Toll-Like Receptors

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Cathepsin E, an intracellular aspartic proteinase, is predominantly localized in the endosomal compartments of immune system cells. In the present study, we investigated the role of cathepsin E in immune defense systems against bacterial infection. Cathepsin E–deficient (*CatE*$^{-/-}$) mice showed dramatically increased susceptibility to infection with both the Gram-positive bacterium *Staphyrococcus aureus*, and the Gram-negative bacterium *Porphyromonas gingivalis* when compared with syngeneic wild-type mice, most likely due to impaired regulation of bacterial elimination. Peritoneal macrophages from *CatE*$^{-/-}$ mice showed significantly impaired tumor necrosis factor-α and IL-6 production in response to *S. aureus* and decreased bactericidal activities toward this bacterium. Moreover, the cell surface levels of Toll-like receptor-2 (TLR2) and TLR4, which recognize specific components of Gram-positive and -negative bacteria, respectively, were decreased in *CatE*$^{-/-}$ macrophages, despite no significant difference in the total cellular expression levels of these receptors between the wild-type and *CatE*$^{-/-}$ macrophages, implying trafficking defects in these surface receptors in the latter. These results indicate an essential role of cathepsin E in immune defense against invading microorganisms, most probably due to regulation of the cell surface expression of TLR family members required for innate immune responses.

Key words: aspartic proteinase, cathepsin E, knockout, macrophage, Toll-like receptors, trafficking.

Abbreviations: BLP, bacterial lipoprotein; *CatE*$^{-/-}$, cathepsin E–deficient; CFU, colony forming unit; Imiquimod, imidazoquinoline; LAMP, lysosome-associated membrane protein; LIMP, lysosomal integral membrane protein; LGP, lysosomal membrane glycoprotein; LPS, lipopolysaccharide; MALP-2, macrophage-activating lipopeptide-2; PEI, phycoerythin intensity; PGN, peptidoglycan; poly I:C, polyinosinic-polycytidylic acid; PBS, phosphate-buffered saline; TLR, Toll-like receptor.

Cathepsin E [EC 3.4.23.34] is an intracellular aspartic proteinase of the pepsin superfamily, which is predominantly expressed in immune system cells including antigen-presenting cells and lymphocytes (*1*–*3*). We have recently generated cathepsin E–deficient (*CatE*$^{-/-}$) mice which spontaneously develop atopic dermatitis-like skin lesions when kept under conventional conditions (*4*). Since *CatE*$^{-/-}$ mice do not develop atopic dermatitis-like skin lesions when reared under specific pathogen-free conditions, the development of this disorder in these mice seems to be triggered by some environmental factor(s) such as pathogenic microorganisms. Indeed, a number of studies have shown that patients with atopic dermatitis exhibit impaired innate and acquired immune responses, resulting in increased susceptibility to microorganisms (*5*–*7*). To date, although colonization by *Staphyrococcus aureus* is thought to be one of the most important environmental factors in human atopic dermatitis (*8*–*11*), the molecular mechanism underlying this event remains mostly unknown.

More recently, we also demonstrated that peritoneal macrophages from *CatE*$^{-/-}$ mice exhibited a new form of lysosomal storage disorder manifesting as the accumulation of three major lysosomal membrane sialoglycoproteins, LAMP-1, LAMP-2, and LIMP-2/LGP85, in the cells and an elevated lysosomal pH (–). Since acidic pH is essential for both the maintenance of normal properties of endolysosomal compartments, and the normal processing and targeting of lysosomal proteins (*13*, *14*), and since the elevated lysosomal pH interferes with the maturation and fusion events of the organelles involved (*15*), *CatE*$^{-/-}$ macrophages are presumed to exhibit trafficking defects for both secretory and endocytosed proteins. Indeed, we found the enhanced secretion of soluble lysosomal enzymes by *CatE*$^{-/-}$ macrophages (*12*). Moreover, we found abnormal trafficking of LAMP-1, LAMP-2, and

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LIMP-2/LGP85 in *CatE*$^{-/-}$ macrophages. It is known that LAMPs and LIMP-2/LGP85 are mainly localized in the limiting membranes of lysosomes and late endosomes, but small amounts of these membrane glycoproteins are also detectable on the cell surface (*16–18*). Intriguingly, we found decreased levels of LAMP-1 and LAMP-2 on the surface of *CatE*$^{-/-}$ macrophages, together with the concurrent accumulation of these proteins in intracellular compartments.

Macrophages are known to express a broad range of cell surface receptors to recognize a wide range of specific endogenous and exogenous ligands (*19*). In particular, Toll-like receptors (TLRs) recognize specific components of microorganisms and trigger the activation of innate immunity (*20–22*). To date, more than 10 TLRs have been identified in humans and mice. Among them, TLR2 recognizes peptidoglycan (PGN), a major Gram-positive bacterial cell wall component, whereas TLR4 is essential for the recognition of lipopolysaccharide (LPS), a Gram-negative bacterial cell wall component (*23*). TLR2-deficient mice exhibit impaired cytokine production in response to PGN prepared from *S. aureus* and therefore are highly susceptible to *S. aureus* infection (*24*). Similarly, TLR4-mutated C3H/HeJ mice are highly susceptible to infection with Gram-negative bacteria due to the failure of LPS recognition (*25–27*). Therefore, it is of special interest to determine whether the susceptibility to bacterial infection and to what extent the surface expression levels of TLRs in macrophages are affected by cathepsin E deficiency. If the surface expression of these receptors on *CatE*$^{-/-}$ macrophages is decreased by their trafficking defects, the cellular responses to cytokines and ultimately cellular functions must be impaired. Therefore, to better understand the role of cathepsin E in the endolysosomal system, it is of particular importance for us to determine the effect of cathepsin E deficiency on the *in vivo* mortality as to bacterial infection, and the *in vitro* susceptibility of macrophages to microorganisms and their products. We herein show that cathepsin E deficiency causes increased mortality as to bacterial infection due to the increased persistence in the blood and spleen, and disrupts appropriate immune responses by macrophages and splenocytes, most probably due to a decrease in a wide range of cell surface TLRs receptors. We thus conclude that cathepsin E contributes to the maintenance of homeostasis by participating in host defense mechanisms.

MATERIALS AND METHODS

Materials—PGN from *S. aureus* and LPS from *Eschericha coli* O55:B55 were purchased from Sigma-Aldrich Co. St. Louis, MO, U.S.A. RPMI-1640 was from Gibco-Life Technologies (Invitrogen, Japan). Fluorescence BioParticles of *S. aureus* were from Molecular Probes (Invitrogen, Japan). Anti-mouse TLR2 monoclonal antibodies (Clone 6C2) were purchased from HyCult Biotech. (Uden, Netherlands). Anti-mouse TLR4-MD-2 monoclonal antibodies (Sa15-21) were kindly donated by Dr. Kensuke Miyake of The University of Tokyo. Specific components of the microorganisms used, including bacterial lipoprotein (BLP), polyinosinic-polycytidylic acid (poly I:C), imidazoquinoline (Imiquimod), CpG DNA, and macrophage-activating lipopeptide-2 (MALP-2), were kindly donated by Dr. Kiyoshi Takeda of Kyushu University.

Mice—Wild-type and *CatE*$^{-/-}$ mice with the C57BL/6 genetic background were used as described previously (*4*). Animals were maintained under specific pathogen-free conditions according to the guidelines of the Japanese Pharmacological Society. Animals and all experiments were approved by the Animal and Microbiological Research Committee of the Graduate School of Dental Science, Kyushu University. Infection experiments were performed with age-matched, male *CatE*$^{-/-}$ mice and wild-type littermates.

Bacteria and Infection—Gram-positive bacterium *S. aureus*, the 209P strain, which was donated by Dr. Koji Nakayama of Nagasaki University, was cultured on trypticase soy agar, inoculated with trypticase soy broth, and then incubated for 18 h at 37°C. Gram-negative bacterium *P. gingivalis* W83 was cultured as described previously (*28*). The bacteria were then harvested by centrifugation and resuspended in phosphate-buffered saline (PBS). The concentration of bacterial cells was adjusted spectrophotometrically at 660 nm. In *in vivo* experiments, a viable bacterial solution (0.2 ml) containing *S. aureus* (5×10^8 CFU) or *P. gingivalis* (1×10^9 CFU) was injected intradermally into the back into wild-type and *CatE*$^{-/-}$ mice.

Preparation of Peritoneal Macrophages—Thioglycolate-elicited peritoneal macrophages were isolated as described previously (*29*). Briefly, 8–14-week-old mice were injected peritoneally with 4.05% thioglycolate (2 ml/mouse). Three and half days later, peritoneal exudate cells were isolated from the peritoneal cavity by washing with PBS. The cells were incubated with RPMI 1640 medium supplemented with 10% penicillin (50 units/ml) and streptomycin (50 μg/ml) at 37°C with 5% CO_2. After incubation for 2 h, non-adherent cells were removed by washing three times with Ca^{2+}/Mg^{2+}-free PBS. MAC-2–positive macrophages comprised more than 95% of the isolated cells.

Determination of the Numbers of S. aureus *in Blood and Other Organs*—Wild-type and *CatE*$^{-/-}$ mice were intravenously injected with *S. aureus* [5×10^7 colony forming units (CFU)] and sacrificed 4 days later. Various organs were dissected out, homogenized, and diluted in 10-fold steps with sterile water containing 0.5% Triton X-100. Blood was also taken and lysed in water containing 0.5% Triton X-100. CFU were determined by plating serially diluted organ homogenates and blood on trypticase soy agar, followed by incubation for 24 h at 37°C.

Cell Staining and Flow Cytometry—Each cell suspension (2×10^5 cells/100 μl) from wild-type and *CatE*$^{-/-}$ mice was incubated on ice for 15 min with primary antibodies appropriately diluted with PBS containing 2.5% FBS and 0.01% NaN_3 (buffer A). Samples were preincubated with Fc Block (anti–mouse CD16/CD32 antibody) (BD Pharmigen), and then the primary antibodies, i.e., biotinylated rat anti–mouse TLR2 (Clone 6C2), biotinylated rat anti–mouse TLR4-MD-2 (Sa15-21), biotinylated isotype control anti–rat IgG2a, or IgG2b (BD Pharmingen), for 15 min on ice. After washing with buffer A, the stained cells were incubated with phycoerythin-streptoavidin (BD Pharmingen) for 15 min on ice. Permeabilized cells were prepared by treatment with 0.03% saponin in the same buffer for

15 min on ice. Flow cytometric analyses were performed on a Beckman Coulter Epics XL cytometer.

Metabolic Labeling—Metabolic labeling experiments were performed as described previously (*30*). In brief, wild-type and *CatE*$^{-/-}$ macrophages were preincubated for 1 h at 37°C in DMEM supplemented with 10% FBS in the absence of methionine and then labeled for 24 h with [^{35}S]methionine (100 μCi/ml) (1.5 ml/plate). Then, the cells were separated from the medium by centrifugation, washed twice with PBS, and lysed by ultrasonication for 1 min in PBS containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.02 % sodium azide, and a proteinase inhibitor cocktail (containing antipain, chymostatin, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride; 1 mg/ml each).

Immunoprecipitation and Gel Electrophoresis—The cell lysates and media were mixed with 40 μl of Pansorbin for 1 h at 4°C to prevent nonspecific binding to IgG–protein A beads, and then centrifuged at 6,500 × *g* for 30 min. The supernatant fractions were incubated with 10 μl of the monoclonal antibody to TLR2 or TLR4/MD2 at 37°C for 10 min, and then stored at 4°C for 16 h. The mixtures were further incubated with 20 μg of goat anti-rat antibodies at 37°C for 10 min and then stored at 4°C for 3 h. Immune complexes were adsorbed onto protein A–Sepharose beads (50% gel suspension) at 4°C for 3 h with gentle agitation, followed by three washes with 0.1% SDS/0.1% Triton X-100/200 mM EDTA/10 mM Tris-HCl (pH 7.5). The immunprecipitates were washed another three times with the same buffer containing 1 M NaCl and 0.1% sodium lauryl sarcosinate, and twice with 5 mM Tris-HCl (pH 7.0). The beads were boiled for 5 min at 100°C with 50 ml of 0.1% SDS/0.5 mM EDTA/5% sucrose/5mM Tris-HCl (pH 8.0) containing 2-mercaptoethanol. SDS-PAGE and fluorography were performed as described previously (*31*).

Bactericidal Activity of Macrophages—The bactericidal activity of macrophages was determined by means of the colony forming unit (CFU) assay. After preincubation for 90 min at 37°C followed by washing, the cells (2×10^5) were incubated with *S. aureus* (2×10^7 CFU) in RPMI-1640 medium containing 10% FBS in the absence of antibiotics for 30 min at 37°C in a 5% CO_2 incubator to allow phagocytosis of the bacteria. After adding penicillin G (100 U/ml) and streptomycin (300 μg/ml), the cells were incubated for the indicated times and then removed with a cell scrapper (Nalge Nunc International, Naperville, IL, USA). After centrifugation, the cells were lysed, serially diluted, plated on trypticase soy agar plates, and then incubated overnight at 37°C. The numbers of aerobic colonies were determined.

Determination of Cytokine Production by Macrophages and Splenocytes—The cytokine levels in culture supernatants were determined by enzyme-linked immunosorbent assaying using specific antibodies.

Phagocytotic Activity of Macrophages—The phagocytotic activity of macrophages (1×10^5 cells) was determined by incubation with fluorescence BioParticles of *S. aureus* (10 mg/ml) for 30 or 60 min. The internalized particles in cells were analyzed by flow cytometry.

Statistical Analysis—The statistical significance of differences in cytokine production was determined with Student's *t* test. Kaplan-Meier plots were made, and the log-rank test was used to determine the differences in bacterial viability between wild-type and *CatE*$^{-/-}$ mice. The statistical significance of differences in the numbers of bacteria in the blood and various organs were also determined with the Mann-Whitney *U* test.

RESULTS

Increased Susceptibility to Bacterial Infection in CatE$^{-/-}$ *Mice*—To evaluate the involvement of cathepsin E in inflammation and host defense mechanisms during bacterial infection, we determined the susceptibility of *CatE*$^{-/-}$ mice and their wild-type littermates to infection with the viable Gram-positive bacterium *S. aureus* (5×10^8 CFU) or the viable Gram-negative bacterium *P. gingivalis* (1×10^9 CFU), their survival being monitored for 7 days (Fig. 1). Following infection with *S. aureus*, *CatE*$^{-/-}$ mice exhibited increased mortality compared with wild-type mice (30% survival for *CatE*$^{-/-}$ mice *vs.* 80% survival for wild-type mice). Following infection with *P. gingivalis*, all *CatE*$^{-/-}$ mice succumbed between days 2 and 7 after infection, while wild-type littermates died from day 2 but 80%

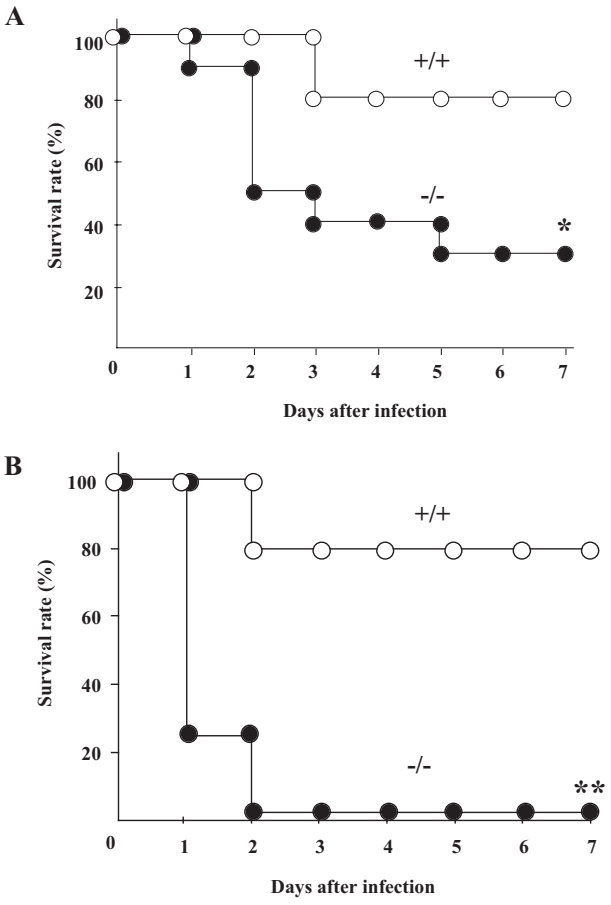

Fig. 1. **Increased susceptibility of *CatE*$^{-/-}$ mice to infection with *S. aureus* or *P. gingivalis*.** Ten *CatE*$^{-/-}$ mice, as well as wild-type littermates, were inoculated intradermally into the back with *S. aureus* (5×10^8 CFU) (A) and intraperitoneally with *P. gingivalis* (1×10^9 CFU) (B), and then their survival was monitored daily up to 7 days. *$P < 0.05$, **$P < 0.01$ *versus* the corresponding values for the wild-type littermates, with the log-rank test.

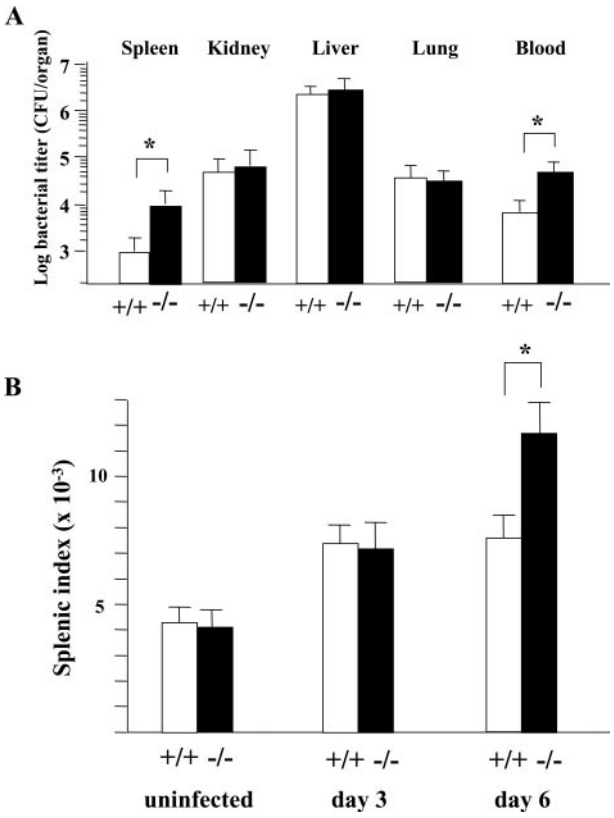

Fig. 2. **The numbers of *S. aureus* in blood and various organs of wild-type and *CatE*$^{-/-}$ mice at 4 days after infection.** (A) Five *CatE*$^{-/-}$ mice, as well as wild-type littermates, were inoculated intravenously with *S. aureus* (5×10^7 CFU). At 4 days after infection, blood and various organs were prepared, and then CFU numbers were determined by plating serially diluted blood and organ homogenates on trypticase soy agar, followed by incubation for 24 h at 37°C. Data are means ± SD of the values for three independent experiments. *$P < 0.05$ *versus* the corresponding values for the wild-type mice, with the Mann-Whitney *U* test. (B) The splenic index was also determined with spleens prepared from wild-type or *CatE*$^{-/-}$ mice at days 3 and 6 after infection with *S. aureus* (5×10^7 CFU) under the same conditions as given in (A). Data are means ± SD of the values for three independent experiments. *$P < 0.05$ *versus* the corresponding values for the wild-type mice, with the unpaired Student's *t* test.

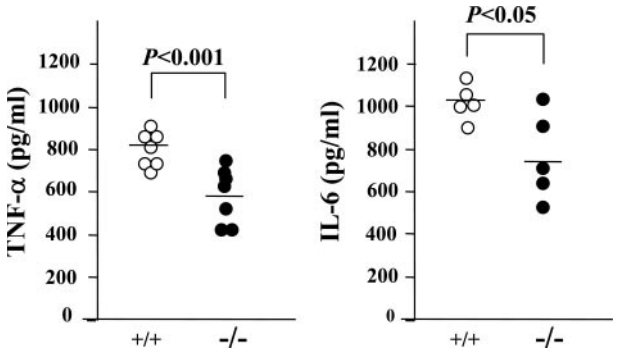

Fig. 3. **Cytokine production by macrophages and splenocytes derived from wild-type or *catE*$^{-/-}$ mice in response to *S. aureus* infection.** Peritoneal macrophages (1×10^5 cells) from *CatE*$^{-/-}$ mice and wild-type littermates were cultured for 24 h with live *S. aureus* (1×10^7 CFU), and then the amounts of TNF-α and IL-6 released into culture supernatants were measured by ELISA with the respective antibodies. The data are means ± SD for five independent experiments. *$P < 0.05$, **$P < 0.001$ *versus* the corresponding values for the wild-type macrophages, with the unpaired Student's *t* test.

survival at 7 days after infection. These results indicate the remarkably higher susceptibility of *CatE*$^{-/-}$ mice to both *S. aureus* and *P. gingivalis* infection compared with wild-type littermates. To determine whether or not the increased susceptibility of *CatE*$^{-/-}$ mice to bacterial infection was due to impaired bacterial elimination, we determined the bacterial load in blood and organ homogenates after infection with *S. aureus*. While there were no significant differences in the numbers (CFU) of the bacterium in any of the tissues tested and blood between wild-type and *CatE*$^{-/-}$ mice at 2 days (data not shown), *CatE*$^{-/-}$ mice exhibited significantly higher bacterial numbers in the spleen and blood, but not the kidney, liver or lung, at 4 days after infection (Fig. 2A). We also observed a remarkable splenomegaly in *CatE*$^{-/-}$ mice between days 3 and 6 after infection in this connection (Fig. 2B). When infected with *S. aureus*, the splenic index (spleen weight per body weight) in *CatE*$^{-/-}$ mice was about 1.5-fold higher than that in wild-type littermates at 6 days after infection, despite no difference between the two animal groups at 3 days after infection (Fig. 2B). Given that cathepsin E is highly abundant in the spleen, as in blood cells, the results suggest critical roles of this enzyme in inflammatory responses and host defense against bacterial infection.

Decreased Cytokine Production in CatE$^{-/-}$ *Macrophages and* CatE$^{-/-}$ *Splenocytes in Response to* S. aureus—We next analyzed the effect of viable *S. aureus* infection on the IL-6 and TNF-α release by peritoneal macrophages derived from wild-type and *CatE*$^{-/-}$ mice. The macrophages derived from *CatE*$^{-/-}$ mice exhibited the decreased production of both IL-6 and TNF-α in response to *S. aureus* infection as compared with the corresponding wild-type cells (Fig. 3). Furthermore, we found that *CatE*$^{-/-}$ splenocytes, when treated with heat-killed *S. aureus*, markedly decreased the production of IFN-γ and IL-10, as compared with the wild-type cells (data not shown).

Normal Phagocytosis of CatE$^{-/-}$ *Macrophages*—Phagocytosis of infected bacteria and subsequent intracellular killing by macrophages act as defense systems during the early phase of bacterial infection (*32, 33*). To determine whether or not the phagocytic activity of macrophages depended on cathepsin E functions, we determined the uptake of fluorescene-labeled *S. aureus* particles by flow cytometry. As demonstrated in Fig. 4, there was no significant difference in the uptake of bacterial particles between wild-type and *CatE*$^{-/-}$ macrophages, implying that a general mechanism of phagocytosis is not impaired by cathepsin E deficiency.

Decreased Bactericidal Activity of CatE$^{-/-}$ *Macrophages*—The bactericidal activity of macrophages is thought to be a key defense mechanism against invading bacteria (*34, 35*). We thus determined whether or not the bactericidal activity of macrophages against *S. aureus*

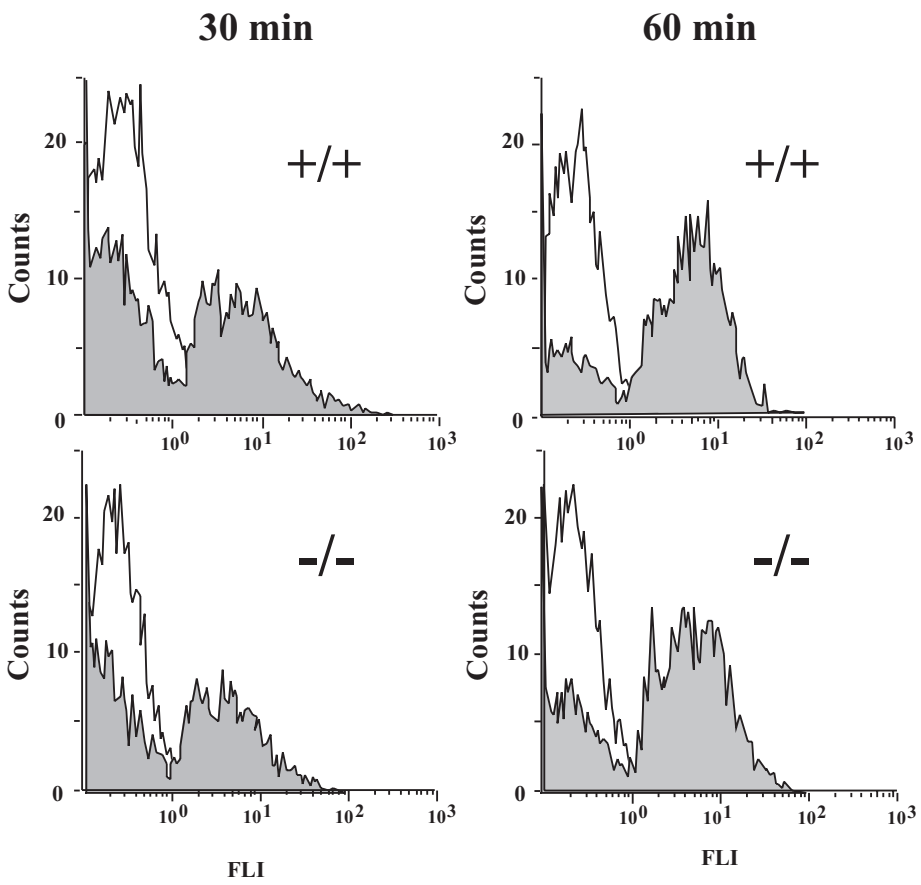

Fig. 4. **Phagocytic activity of wild-type and *catE*$^{-/-}$ macrophages toward *S.* aureus BioPaticles.** Peritoneal macrophages (1×10^6 cells) derived from wild-type and *CatE*$^{-/-}$ mice were cultured with fluorescein *S. aureus* BioPaticles (10 mg/ml) for 30 min or 60 min. The internalized particles were analyzed with a flow cytometer. Data are representative of three independent experiments.

depended on cathepsin E. To this aim, macrophages were preincubated with viable *S. aureus* for 30 min and then further incubated for various times without the bacteria. After lysis of the cells, the numbers of aerobic colonies on agar plates were determined. *CatE*$^{-/-}$ cells exhibited significantly higher bacterial numbers at 4 and 6 h after infection when compared with wild-type cells, although both cell types showed a time-dependent decrease in the bacterial number (Fig. 5). Notably, *CatE*$^{-/-}$ macrophages showed 2.5-fold higher bacterial numbers than wild-type cells at 6 h. These results indicate that cathepsin E deficiency reduces the bactericidal activity of macrophages.

Cell Surface Expression of TLR2 and TLR4 in Wild-Type and CatE$^{-/-}$ *Macrophages*—We previously found that *CatE*$^{-/-}$ macrophages exhibit trafficking defects, most probably due to lysosomal pH (*12*). Given the high expression of cathepsin E in the endolysosomal compartments of antigen-presenting cells, we thus evaluated the cell surface expression of TLR2 and TLR4 on macrophages from *CatE*$^{-/-}$ mice and wild-type littermates by flow cytometry analysis. As shown in Fig. 6A, *CatE*$^{-/-}$ cells showed significant decreases in the proportions of both TLR2- and TLR4-positive cells compared with wild-type cells. However, these differences were abrogated with permeabilized cells that had been treated with saponin (Fig. 6B), indicating that the total levels of expression of TLR2 and TLR4 were comparable between the two cell types.

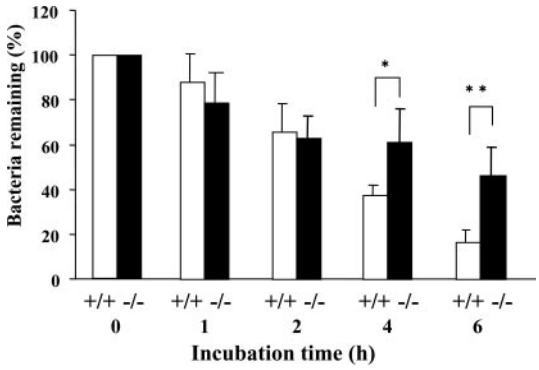

Fig. 5. **Bactericidal activity of wild-type or *catE*$^{-/-}$ macrophages toward *S. aureus*.** Peritoneal macrophages (1×10^6 cells) derived from wild-type and *CatE*$^{-/-}$ mice were preincubated for 90 min and then cultured with *S. aureus* (1×10^8 CFU) for 30 min. After washing, the cells were incubated and harvested at the indicated times. Then the cells were lysed and cultured on trypticase soy agar plates. The numbers of aerobic colonies were determined and expressed as percentages of those at 0 time. The data are means ± SD for five independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ *versus* the corresponding values for the wild-type macrophages, with the unpaired Student's *t* test.

To further substantiate this, both wild-type and *CatE*$^{-/-}$ macrophages were metabolically labeled with [^{35}S]methione for 24 h, and the cell lysates were immunoprecipitated with specific monoclonal antibodies for TLR2

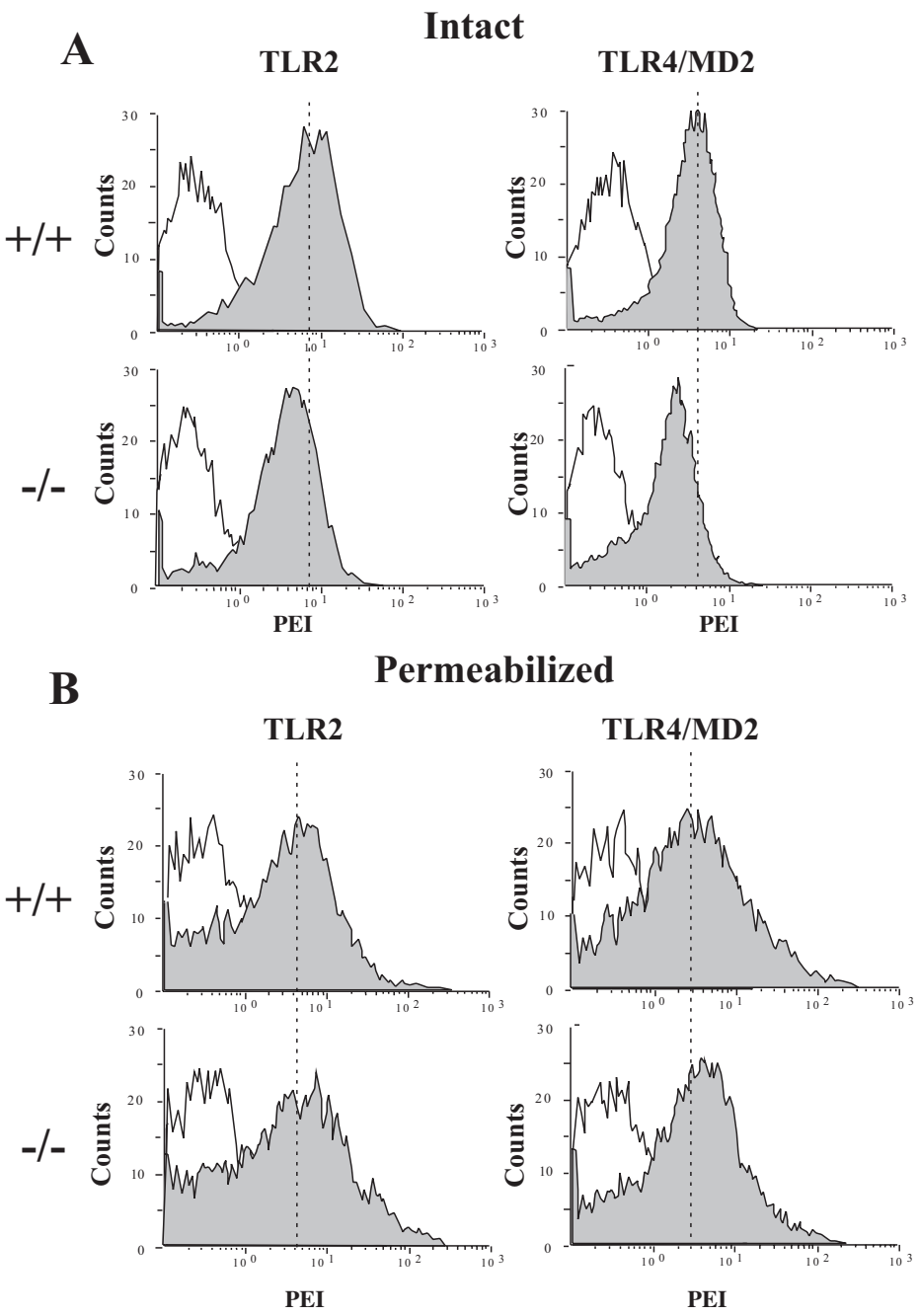

Fig. 6. **Flow cytometry analysis of TLR2 and TLR4 expression in wild-type or *CatE*$^{-/-}$ macrophages.** (A) Macrophages (2×10^5 cells) from wild-type and *CatE*$^{-/-}$ mice were stained for cell surface TLR2 and TLR4 with the respective antibodies and then analyzed by flow cytometry. Data are representative of five independent experiments. (B) The cells were permeabilized with saponin and then stained for determination of the total expression levels of TLR2 or TLR4. Data are representative of five independent experiments.

and TLR4/MD2, and then analyzed by SDS-PAGE and fluorography. There were no significant differences in the cellular levels of TLR2 and TLR4 between wild-type and *CatE*$^{-/-}$ macrophages (Fig. 7). Consistent with these findings, semi-quantitative RT-PCR analysis revealed no significant differences in the mRNA levels of TLR2 and TLR4 between wild-type and *CatE*$^{-/-}$ macrophages (data not shown). These results thus indicate that, despite no effect on their biosynthetic processes, the *CatE*$^{-/-}$ deficiency causes diminished transport of TLR2 and TLR4 to the cell surface. Similar to the previous finding that *CatE*$^{-/-}$ macrophages exhibited trafficking defects in soluble lysosomal proteins, the decreased transport of TLR2 and TLR4 to the cell surface is most likely due to trafficking defects in these cells. The present results thus suggest that the decreased expression of cell surface TLR2 and TLR4 could be responsible for the deficient anti-bacterial defense in *CatE*$^{-/-}$ mice.

Decreased Cytokine Production in CatE$^{-/-}$ *Macrophages in Response to Various Stimuli*—To evaluate the importance of cathepsin E during responses to various specific components of microorganisms, we determined the production of IL-6 and TNF-α by wild-type and *CatE*$^{-/-}$ macrophages at 24 h after stimulation with specific components of microorganisms.

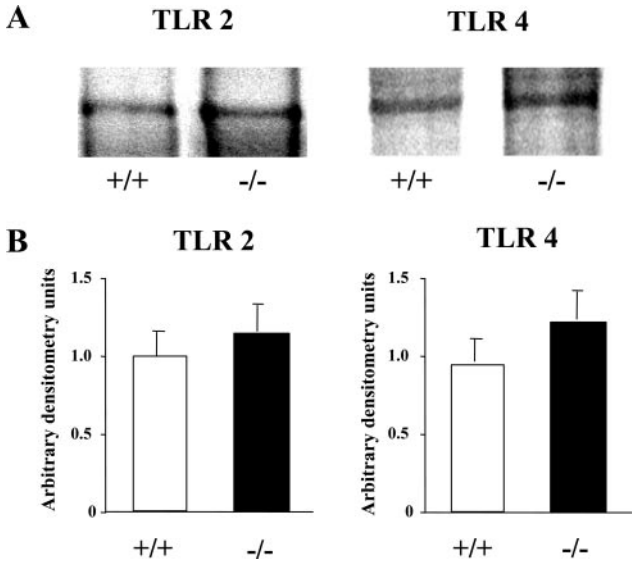

Fig. 7. **Biosynthesis and expression of TLR2 and TLR4 in wild-type and *CatE*$^{-/-}$ macrophages.** (A) Macrophages from wild-type and *CatE*$^{-/-}$ mice were metabolically labeled with [^{35}S]methionine for 24 h and then harvested by centrifugation. Then, the cells were lysed, and the resultant cell lysates were immunoprecipitated with anti–mouse TLR2 or TLR4 mAb, and subjected to SDS-PAGE and fluorography. Data are representative of four independent experiments. (B) Densitometric analysis of the immunoprecipitated TLR and TLR4. The data are means ± SD for four independent experiments.

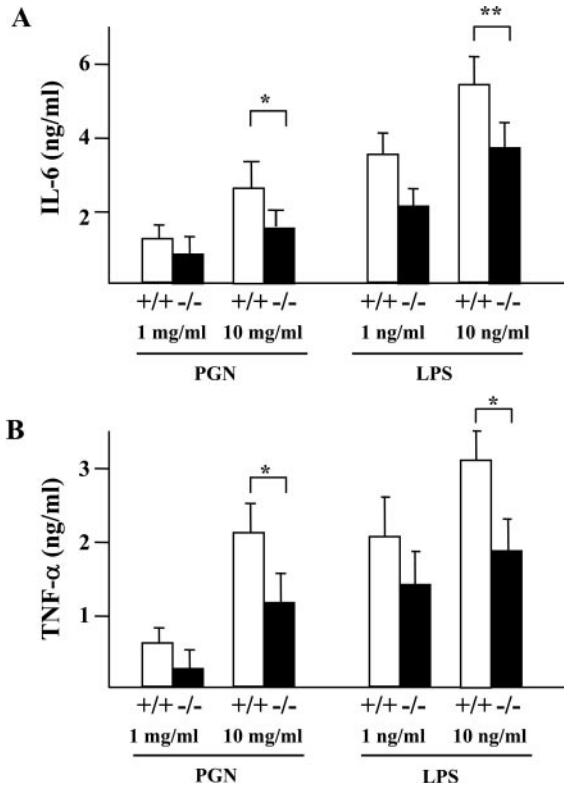

Fig. 8. **Cytokine production by wild-type or *CatE*$^{-/-}$ macrophages in response to specific ligands for TLR2 and TLR4.** Peritoneal macrophages (1×10^5 cells) from wild-type and *CatE*$^{-/-}$ mice were stimulated for 24 h with the indicated concentrations of LPS (the TLR4 ligand) or PGN (the TLR2 ligand), and then the amounts of IL-6 and TNF-α released into culture supernatants were measured by ELISA. The data are means ± SD for five independent experiments. *$P < 0.05$, **$P < 0.01$ *versus* the corresponding values for the wild-type macrophages, with the unpaired Student's *t* test.

The IL-6 and TNF-αlevels in the culture supernatants after stimulation with either the TLR2 ligand PGN (10 mg/ml) or the TLR4 ligand LPS (10 ng/ml) were significantly decreased in *CatE*$^{-/-}$ macrophages as compared with in wild-type cells (Fig. 8), although stimulation with lower concentrations of the ligands (1 mg/ml PGN and 1 ng/ml LPS) induced no statistically significant difference between the two cell types. These results were in fair agreement with the data indicating decreased expression levels of TLR2 and TLR4 (Fig. 7).

To further characterize the effects of other specific components of microorganisms on IL-6 and TNF-αrelease by wild-type and *CatE*$^{-/-}$ macrophages. As shown in Fig. 9, *CatE*$^{-/-}$ macrophages exhibited significantly decreased release of both cytokines in response to the TLR3 ligand Poly I:C and the TLR6 ligand MALP2. *CatE*$^{-/-}$ macrophages also tended to exhibited decreased, if not statistically significant, cytokine release when compared with wild-type cells in response to other ligands, including the TLR1 ligand BLP, the TLR7 ligand Imiquimod, and the TLR9 ligand CpG DNA, under the conditions used. Previous studies have demonstrated that TLR1, TLR2, TLR4, TLR5, and TLR6 are predominantly expressed on the cell surface, while TLR3, TLR7, and TLR9 are primarily localized in intracellular compartments (*36–38*). Therefore, it seems likely that the cathepsin E deficiency also causes trafficking defects in total endocytotic pathways.

DISCUSSION

To our knowledge, this is the first study indicating a critical role of cathepsin E in innate immune responses to infection with Gram-positive and -negative bacteria using *CatE*$^{-/-}$ mice. *CatE*$^{-/-}$ mice exhibited higher mortality compared with wild-type littermates after inoculation with either *S. aureus* or *P. gingivalis*. This may be associated with that the numbers of bacterial cells in the blood and spleen were higher in *CatE*$^{-/-}$ mice compared with in wild-type mice. This impaired bacterial elimination in the blood and spleen of *CatE*$^{-/-}$ mice is more likely associated with its abundant distribution in the blood and spleen cells, and it seems to selectively impair effecter mechanisms responsible for controlling intracellular bacteria. The splenomegaly found in *CatE*$^{-/-}$ mice after *S. aureus* inoculation additionally suggests the importance of cathepsin E in splenic functions. We have previously demonstrated that *CatE*$^{-/-}$ mice spontaneously develop atopic dermatitis-like skin lesions when reared under conventional conditions, but not specific pathogen–free conditions (*4*). Atopic dermatitis is a chronic inflammatory skin disease complicated by continuous bacterial infections, particularly with *S. aureus* (*8–11*). Although the precise mechanism underlying the development of atopic dermatitis–like skin lesions in *CatE*$^{-/-}$ mice remains unclear, the innate immunity system that senses invasion

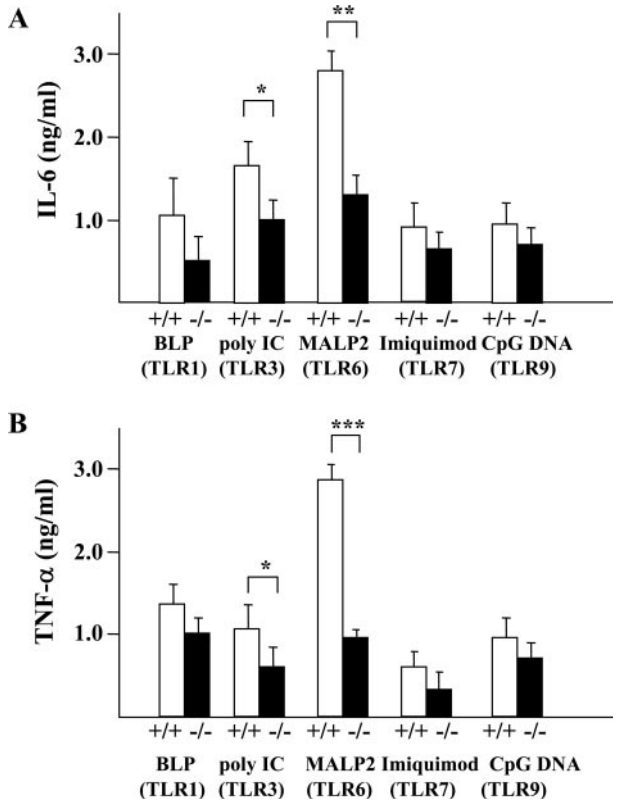

Fig. 9. **Cytokine production by wild-type or CatE$^{-/-}$ macrophages in response to specific microbial components for other TLRs.** Peritoneal macrophages (1×10^5 cells) from wild-type and *CatE*$^{-/-}$ mice were stimulated for 24 h with 10 μg/ml BLP (the TLR1 ligand), 10 μg/ml poly I:C (the TLR3 ligand), 100 ng/ml MALP-2 (the TLR6 ligand), 10 μg/ml imiquimod (the TLR7 ligand), or 100 nM CpG-DNA (the TLR9 ligand). The amounts of IL-6 and TNF-α released into culture supernatants were measured by ELISA. The data are means ± SD for five independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *versus* the corresponding values for the wild-type macrophages, with the unpaired Student's *t* test.

by microorganisms is most probably impaired in these animals. Therefore, the increased susceptibility of *CatE*$^{-/-}$ mice to infection with *S. aureus* and *P. gingivalis* is most probably due to the reduced bactericidal activity associated with impaired TLR recognition.

Innate recognition of bacteria by TLR family members is especially important for eliminating invading bacteria. In particular, TLR2 and TLR4 play crucial roles in innate immune responses to the Gram-positive and -negative bacteria, respectively (*23–27*). Given that TLRs are mainly expressed on myelomonocytic cells, the increased mortality of *CatE*$^{-/-}$ mice after infection with *S. aureus* and *P. gingivalis* is more likely associated with impaired responsiveness of TLR2 and TLR4 on macrophages. We herein demonstrated that, while the total expression levels of both TLR2 and TLR4 did not differ between wild-type and *CatE*$^{-/-}$ macrophages, the cell surface expression levels of these receptors were significantly decreased on *CatE*$^{-/-}$ macrophages. These results thus suggest impaired intracellular trafficking of both receptors to the plasma membranes. Our previous study demonstrated trafficking defects in soluble lysosomal proteins to lysosomes in *CatE*$^{-/-}$ macrophages, probably due to elevated lysosomal pH (*12*). Since an elevated lysosomal pH interferes with the maturation and fusion events of the organelles involved (*13–15*), the elevated lysosomal pH in *CatE*$^{-/-}$ macrophages is more likely to induce impaired trafficking events in both the exocytotic and endocytotic pathways. Indeed, the cell surface expression levels of LAMP-1, LAMP-2, chemokine receptor-2, and formyl peptide receptors were significantly lower in *CatE*$^{-/-}$ macrophages than in wild-type cells (Tsukuba et al., unpublished data). Therefore, the decreased cell surface expression of TLR2 and TLR4 is most probably due to their impaired trafficking to the plasma membranes.

Upon ligation of TLRs to specific components of microorganisms, macrophages produce various proinflammatory cytokines and thereby contribute to cellular activation of immune responses. We reported herein that *CatE*$^{-/-}$ macrophages reduced the production of IL-6 and TNF-α in response to a wide variety of ligands in microorganisms, including the TLR2 ligand PGN, the TLR4 ligand LPS, and the TLR6 ligand MALP2, when compared with wild-type cells. It was surprising that *CatE*$^{-/-}$ macrophages were 2-fold more susceptible to the TLR3 ligand poly I:C compared with wild-type cells. Given its preferential localization in endosomal compartments, the decreased responsiveness of *CatE*$^{-/-}$ macrophages to this ligand implies the impairment of early interactions between phagosomes and endosomes, although the phagocytosis mechanism appeared not to be impaired in *CatE*$^{-/-}$ macrophages. Therefore, the mechanism underlying impaired responsiveness to the TLR3 ligand remains to be elucidated.

Our study also demonstrated the decreased bactericidal activity of *CatE*$^{-/-}$ macrophages toward intracellular bacteria, compared with wild-type cells. Overall, it is plausible to speculate that cathepsin E deficiency in macrophages influences bacterial killing processes after phagocytosis. Given the impairment of fusion between bacteria-loaded phagosomes with lysosomes in macrophages from TLR2 and TLR4 doubly deficient mice followed by TLR signaling defects (*39*), the decreased bactericidal activity of *CatE*$^{-/-}$ macrophages is more likely due to impairment of the phagosome–lysosome transport system. In addition to the abnormality of TLR signaling systems, *CatE*$^{-/-}$ macrophages exhibited a marked decrease in the potential to degrade endogenous and exogenous proteins in endolysosomal compartments (Tsukuba et al., unpublished data). Overall, the present study indicates that cathepsin E plays a crucial role in host defense against invading microbial pathogens via not only TLR signaling pathways but also endolysosomal degradation of intracellular bacteria.

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